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The objective of this project is to genetically engineer a radiometal binding site in a human antibody constant region, for eventual use in tumor radioimmunotherapy. Our initial design was based on molecular modeling and used a humanized antibody Fab fragment expressed in *E. coli*. The engineered binding site, consisting of 5 point mutations in the human Cκ domain, destabilized the recombinant protein, leading to proteolysis. We have attempted to address this problem by using expedited isolation procedures, modifying the binding site design, and expressing the protein within protease-negative host strains.

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Jefferson Foote 10 Aug 98

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Introduction

The isotope Yttrium-90 has decay properties (β-emission, path length <1 cm in tissue) that would make it ideal for radioimmunotherapy. However, stable attachment of yttrium to antibodies that could target the isotope to a tumor have been problematical. The current best technology is to use a synthetic bifunctional chelator to bind the yttrium to the antibody. However, these synthetic molecules when bound to an immunoglobulin act as classical haptens, and can induce an immune response in patients. Such an immune response, directed to the radiopharmaceutical through the chelator, causes rapid inactivation and elimination of the administered therapeutic agent.

Our proposal was to genetically engineer an yttrium binding site in a constant domain of a human immunoglobulin. The site would be located away from the surface of the protein, and would be composed of charged amino acid side chains, rather than an exogenous organic molecule, hence would not be immunogenic.

At the start of the funding period we had engineered an antibody Fab fragment with a putative yttrium binding site in the human Cκ domain, and had expressed it in *E. coli*. We had expected, and had found, that this protein would be very difficult to make, because of instability introduced by burying charged residues in the interior of a protein folding domain. Extensive proteolysis during expression suggested that the recombinant protein had difficulty folding. We had prepared approximately 1 mg of intact protein from a 20 L fermentor run. Our initial efforts to demonstrate yttrium binding were unsuccessful, and exhausted the initial supply of protein.

Body

We decided to attack the protein folding problem directly, using four strategies:

- Fine-tune Fab expression and isolation.
- Use protease-negative mutant host strains
- Redesign the yttrium binding site.
- Co-express genes that promote disulfide bond formation

Fine-tune Fab expression and isolation. Biochemical steps in the protein isolation procedure were extensively optimized, emphasizing speed, low temperature, and addition of EDTA and protease inhibitors. We exhaustively explored rapid isolation on affinity media, including peptostreptococcal protein L (1) and immobilized anti-human Cκ monoclonal antibodies 141PF11 and HP6053 (2). These modifications improved recovery of intact protein from small-scale (50 ml) cultures, but could not successfully be applied on a fermentor scale.

Use protease-negative mutant host strains. We obtained several *E. coli* strains mutant in one or more of the protease genes ompT, degP, clpP, and lon. These strains are listed in Table I. Vector constructs carrying Fab with the engineered Cκ sequence and a control with the wild-type sequence were transferred to the mutant strains, and the expression and rapid isolation procedures were applied. Surprisingly, use of the mutant strains gave no improved recovery of intact Fab.

Table I. Protease-negative E. coli host strains.

Strain	Relevant protease mutations	Reference
SA2817	ompT degP	(3)
SG22094	lon, clpP	(4)
UT5600	ompT	(5)

Redesign the yttrium binding site. Our designed metal binding site had 4 charged amino acid side chains placed with a cubic geometry at the heart of a kappa constant region domain. This charge density obviously would destabilize the domain, but our expectation was that metal chelation would lend some stability by partially neutralizing these charges. To reduce the thermodynamically unfavorable effect of burying charges in a hydrophobic protein domain, we redesigned the metal binding site to contain 2 charged residues. Sequence modifications tested are shown in Table II. However, two alternative designs failed to increase the yield of intact protein.

Table II. Mutated residues in the yttrium binding site designs tested.

Construct	115	136	144	146	196
Control (wild type Ck)	V	L	A	V	V
HuLys Y	E	D	E	E	A
HuLys Y2	E	L	E	V	A
HuLys Y3	E	L	E	V	S

Co-express genes that promote disulfide bond formation. Work by others has shown the importance of disulfide formation in folding of recombinant immunoglobulin fragments (6, 7). We are currently building into our expression vector the genes for 4 enzymes used for disulfide formation in the periplasm of E. coli. Our presumption is that increased gene product copy number will make protein folding more rapid, hence channel more recombinant Fab down this pathway, rather than a proteolytic pathway. At present we have acquired or cloned by PCR the genes listed in the table. Construction is in progress of a plasmid vector that contains all four genes with their endogenous control regions, as well as sequences for Fab expression. We do not yet have data on how this extra machinery will affect production of intact Fab.

Table III. Disulfide metabolism genes incorporated into expression construct.

Gene	Function	Reference
dsbA	Oxidize protein thiols to disulfides	(8)
dsbB	Regenerate (reduce) dsbA	(9)
dsbC	Disulfide isomerase	(10, 11)
dipZ	Maintain dsbC in reduced (thiol) form	(12, 11)

Conclusions

The major obstacle in this project is proteolytic degradation of recombinant Fab, due to the destabilizing effect of the engineered radiometal binding site. Building the binding site sequentially, one amino acid residue at a time, was not a successfully strategy for remediating this instability. Expedited procedures for biochemical isolation of recombinant Fab are necessary, but not sufficient, for adequate yields of protein. These methods must be accompanied by genetic modifications of the host strain and/or vector. Use of host strains with single or double protease mutations did not improve expression. A strain containing complete protease knockout (13) may be necessary.

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